AMENDMENTS TO THE SPECIFICATION

Please replace the original abstract with the following paragraph:

The invention provides oxygen-resistant iron-hydrogenases ([Fe]-hydrogenases) for use in the production of H_2. Methods used in the design and engineering of the oxygen-resistant [Fe]-hydrogenases are disclosed, as are the methods of transforming and culturing appropriate host cells with the oxygen-resistant [Fe]-hydrogenases ~~hyrdogenases~~. Finally, the invention provides methods for utilizing the transformed, oxygen insensitive, host cells in the bulk production of H_2 in a light catalyzed reaction having water as the reactant.

Please replace paragraph [0003] with the following replacement paragraph:

[0003] Hydrogen (H_2) ~~($H2$)~~ is becoming an attractive alternative energy source to fossil fuels [[m]] due to its clean emissions and potential for cost effective production by microorganisms. As such, microorganisms that metabolize H_2 are being investigated for their potential use in H_2-production. A microorganism of particular interest for H_2 production is the green alga, *Chlaydomonas* reinhardtii, which is able to catalyze light-dependent, H_2 production utilizing water as a reductant. Ghiradi et al., (2000) Trends Biotech. 18(12):506-511; Melis et al., (2001) Plant Physiol. 127:740-748. The benefits of using an algal system for H_2-production include the use of renewable substrates (light and water) and its potential cost-effectiveness. Melis A, Int. J. Hyd. Energy 27:1217-1228. As such, there is a great deal of interest in optimizing H_2-production by green algae to maximize the potential benefit as an alternative energy source.

Please replace paragraph [0012] with the following replacement paragraph:

[0012] FIG. 1A shows a protein alignment using PILEUP/GENEDOC program (CaI is represented by SEQ ID NO: 1; CpI is represented by SEQ ID NO: 2; Dd is represented by SEQ ID NO: 3; CrHydA2 is represented by SEQ ID NO: 4; CrHydA1 is represented by SEQ ID NO: 5). Amino acid residues highlighted in black represent identities between at least 5 of the iron-hydrogenases, and those highlighted in grey show similarity between at least 5 of the sequences (1A). FIG. 1B shows a theoretical structure of HydA1 using homology modeling to the solved X-ray structure of CpI. The left panel shows an overlay of HydA1 and CpI, with locations of the H_2-channels and the active sites, while the right panel shows the HydA1 structure.

Please replace paragraph [0013] with the following replacement paragraph:

[0013] FIG. 2 shows the protein sequence of HydA1 (SEQ ID NO: 6) aligned to the catalytic core region of CpI (SEQ ID NO: 7). The sequences that form the H_2-channel domain are shaded either gray (similar) or black (identical).

Please replace paragraph [0021] with the following replacement paragraph:
[0021] The following definitions are provided to facilitate understanding of certain terms used herein and are not meant to limit the scope of the present disclosure.

Please replace paragraph [0030] with the following replacement paragraph:
[0030] "Host cell" refers to cells containing a target nucleic acid molecule, for example a heterologous nucleic acid molecule such as a plasmid or other low molecular weight nucleic acid, in which case the host cell is typically suitable for replicating the nucleic acid molecule of interest. Examples of suitable host cells useful in the present invention include bacteria, algae, and yeast. Specific examples of such cells include E. *Coli* DH5α cells, as well as various other bacterial cell sources, for example, the E. Coli strains: DH10b cells, XL1Blue cells, XL2Blue cells, Top10 cells, BB101 cells, and DH12S cells, yeast host cells from the genera including Saccharomyces, Pichia, and ***Kluveromyces***, and green alga, for example, *Chlamydomonas* reinhardtii.

Please replace paragraph [0039] with the following replacement paragraph:
[0039] In a preferred embodiment of the invention, a process for designing and engineering oxygen-resistant iron-hydrogenases has been developed. The engineering scheme targets the structure and or environment of the H_2-channel within the target hydrogenase, which is altered to be more selective in allowing the outward diffusion of hydrogen while simultaneously filtering out [[our]] surface oxygen. Note that size-limited diffusion has been successfilly used to generate filters for commercial use in the separation of gases, including the separation of hydrogen from oxygen. Menoff T. M., (2000) Proc. Hydrogen Program Review.

Please replace paragraph [0044] with the following replacement paragraph:
[0044] As noted above, the present invention provides a model for generating a theoretical structure of a target H_2-channel within a target hydrogenase enzyme. In one embodiment, the theoretical structure is generated by homology modeling (see above) to the solved structure of other known [Fe]-hydrogenases, for example CpI. (see Figure 1A). In some embodiments, the

homology modeling is limited to the known hydrogenase active site and H_2-channel, and in other embodiments the homology modeling can be limited to the known hydrogenase H_2-channel sub-domains. A percent homology of the known hydrogenase (both identity and similarity) can be used to determined residue identity and similarity for the entire enzyme, the active site, the H_2-channel and the H_2-channel sub-domains (see overhead arrows in FIG. 2 and see discussion in previous section above). As such, the present invention provides a known hydrogenase based homology model that gives a reliable approximation of the target hydrogenase structure and H_2-channel environment. In a preferred embodiment, the known hydrogenase is CpI and the target hydrogenase is HydA1. Homology modeling can be performed using Swiss-model software as described in Guex et al. ~~Guex et al~~ {1997} Electrophoresis 18:2714-2723. Note, however, that other like programs can be used in this aspect, as is known in the art, e.g., Modeller program designed by Marti-Renom et al., (2000) Ann. Rev. Biophy, Biomol. Struct. 29:291-325; EsyPred3D designed by Lambert C. et al., (2002) Bioinformatics 18(9):1250-1256.

Please replace paragraph [0047] with the following replacement paragraph:

[0047] Designed oxygen-resistant hydrogenases, having a reduced diameter H_2-channel, are genetically engineered and transformed into target host cells, for example, into C. reinhardtii, and tested for hydrogenase activity in the presence of O_2 via a modified Clark electrode or other known assay(s). In preferred embodiments, the oxygen-resistant hydrogenase is generated via site-directed mutagenesis. For example, to generate HydA1 mutants, the HydA1 gene of pA1ExBle can be mutagenized in vitro using the Quick Change XL Site-Directed Mutagenesis Kit (Stratagene). Host cells that have incorporated the designed enzymes having reduced oxygen sensitivity) can be used to photoproduce H_2 in an oxygen containing environment. Note that these host cells can also be treated with mRNA interference to repress the expression of native hydrogenases, while continuing to allow expression of the inventive engineered hydrogenase(s).

Please replace the heading between paragraphs [0053] and [0054] with the following heading:

Oxygen-Resistant Hydrogenase ~~Hydrogena se~~ Polynucleotides, Vectors and Host Cells

Please replace paragraph [0062] with the following replacement paragraph:

[0062] To facilitate the design and engineering of mutant oxygen-resistant HydA1 enzymes, a theoretical structure of HydA1 was generated by homology modeling to the solved X-ray

structure of Clostridium pasteuraianuin [Fe]-hydrogenase, CpI (FIG. 1B). The theoretical model was generated by homology modeling using Swiss-model software as described by ~~Guex et al.~~ Guex et al., (1997) Electrophoresis 18:2714-2723. The resulting HydAl model was subjected to several rounds of energy minimization using GROMOS. An alignment of the HydAl and CpI amino acid sequences show they share a high degree of homology (45% identity, 58% similarity) within the essential domains, *i.e.*, active site and H_2-channel, that comprise the core region of [Fe]-hydrogenases (see FIG. 2). Stothard P., (2000) BioTechniques 28(6) 1102. Note that the degree of conservation increases for H_2-channel sub-domains, where the two proteins share 62% identity and 92% similarity (FIG. 2, overhead arrows). This high level of sequence identity/similarity shows that the CpI-based HydAl homology model provides a reasonable approximation of the HydAl structure and the H_2-channel environment.

Please replace paragraph [0067] with the following replacement paragraph:

[0067] The above results indicate that modeling of the HydAl structure has revealed a hydrophobic channel extending from the active site to the enzyme surface. This channel would appear to be conserved in other [Fe]-hydrogenases. The channel's secondary structure is mainly a-helical, which suggests that the channel domain is fairly rigid. Perhaps the rigidity of the channel structure helps to prevent its collapse during folding. Volbeda et al., (2002) Int. J. Hyd. Energy 27:1449-1461. Rigidity would also be expected to contribute to conformational stability of the channel in the folded protein, and a static model should give reasonable approximations of shape and size. Our measurements of the HydAl channel demonstrate that it is sufficient in diameter not only to allow for diffusion of the product H_2 but also the larger-sized inhibitors O_2 and CO. Since enzyme inhibition occurs quickly (minutes), following exposure of O_2 (Happe et al., (1994) Eur. J. Biochem. 222:769-774), the channel would not appear to be highly restrictive to inhibitor diffusion, which is in agreement with our analysis. This data illustrates the utility of the present invention for engineering O_2-resistant, [Fe]-hydrogenase by manipulation of residues within the conserved H_2 channel. This modeling approach can be used in enzymes that have one channel or multiple channels to reduce inhibitor access to an enzyme active site.

Please replace paragraph [0073] with the following replacement paragraph:

[0073] To ensure that the HydAl cDNA genomic insert having the V240W mutation was present in the transformed C. reinhardtii, PCR and sequencing was performed on Ble^r transformants. Total genomic DNA was isolated from individual Ble^r transformants using the Plant Genomic

Kit (Qiagen). A total of 0.5 to 1.0 µg of purified genomic DNA was digested with either SacI or EcoRI and used as template in a PCR reaction consisting of the HydA1 internal primers (5'-CACGCTGTTTGGCAT CGACCTGACCATCATG-3' JSEQ ID NO: 8) and 5'-GCCACGGCCACGCGGAATGTGATG CCGCCCC-3' JSEQ ID NO: 9)), 1 unit KOD HotStart polymerase (Novagen), 10 mM $MgSO_4$ [[4]], 25 mM of each dNTP, 2% DMSO (v/v), and water to a total volume of 50 µl. The presence of a HydA1 cDNA genomic insert results in an additional 780 bp HydA1 cDNA product together with the 1120 bp HydA1 genomic product. PCR reactions were run on 1x TAE agarose gels (1.25% agarose w/v), stained with ethidium bromide, and photographed (not shown). The 780 bp band, corresponding to the HydA1 cDNA insert, was purified and sequenced to confirm the presence of V240W mutation. Two Ble^r *C. reinhardtii* clones, mt18 and mt28, were shown to possess the HydA1V240W construct (see FIG. 5).